Universidade de Aveiro Departamento de Biologia
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AVALIAÇÃO DO POTENCIAL GENOTÓXICO DE NANOMATERIAIS DE GRAFENO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, ramo da Biologia, realizada sob a orientação científica da Doutora Helena Cristina Correia de Oliveira, Investigadora de Pós-doutoramento do Departamento de Biologia do Centro de Estudos do Ambiente e do Mar – CESAM e do Instituto de Materiais Aveiro – CICECO da Universidade de Aveiro e sob co-orientação do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar – CESAM da Universidade de Aveiro.

Dedico este trabalho aos meus pais, meu irmão e meu namorado, com todo o meu amor e gratidão.

o júri

Presidente	**Prof. Doutor Mário Guilherme Garcês Pacheco** Professor Auxiliar com Agregação da Universidade de Aveiro
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palavras-chave

Grafeno, Citotoxicidade, Genotoxicidade, linha celular RAW 264.7, Cultura celular

resumo

O Grafeno é constituído por uma única camada de átomos de carbono, estruturada em forma hexagonal com os átomos de carbono ligados por hibridação sp^2 e, desde o seu isolamento as suas propriedades únicas, tais como, grande área superficial, condutividade elétrica e térmica, têm sido bastante estudadas. Os nanomateriais de grafeno e os que são à base de grafeno são atualmente aplicados em várias áreas, tais como medicina, eletrónica, sensores e ambiente. Apesar da ampla aplicação dos nanomateriais de grafeno, as informações sobre os riscos para a saúde humana e o meio ambiente não são ainda bem conhecidas. Consequentemente, é importante investigar e avaliar a potencial toxicidade do grafeno. Esta tese revê as aplicações e a toxicidade da Família dos Nanomateriais de Grafeno (GFNs) em células humanas. Além disso, os ensaios para a avaliação in vitro dos efeitos genotóxicos também foram brevemente revistos. A relevância da avaliação dos potenciais efeitos genotóxicos das GFNs é destacada nesta tese.

keywords Graphene, Citotoxicity, Genotoxicity, RAW264.7 cell line, Cell Culture

abstract

Graphene is a purely carbon-based, honeycomb-structured, one-atom thick layer of carbon atoms, bonded to another by sp^2 hybridization and since its isolation, its unique properties such as high surface area, electrical and thermal conductivities have been widely studied. Graphene and graphene-based nanomaterials are now applied in several fields, as for instance medicine, electronics, sensors and environment. Despite the wide application of graphene nanomaterials, the information on the risks to human health and the environment are not yet well known. Consequently, it is important to investigate and evaluate the potential toxicity of graphene. This thesis revises the applications and toxicity of Graphene-Family Nanomaterials (GFNs) to human cells. Also, the assays for in vitro evaluation of genotoxic effects were also shortly revised. The relevance of the evaluation of potential genotoxic effects of GFNs is highlighted in this thesis.

List of Tables and Figures

List of Acronyms and Abbreviations

BMD – Benchmark Dose

CMBN – Cytokinesis-blocked Micronucleus Assay

CNT – Carbon Nanotubes

Cyt-B – Cytochalasin-B

DMEM – Dulbecco's Modified Eagle's Medium

FLG – Few-layer Graphene

FBS – Fetal Bovine Serum

GFNs – Graphene-Family Nanomaterials

GO – Graphene Oxide

GSH-PX – Glutathione Peroxidase

MMP – Mitochondrial Membrane Potential

MMS – Methyl Methanesulfonate

Mni – Micronuclei

NPs – Nanoparticles

NBUDs – Nuclear Buds

NPBs – Nucleoplasmic Bridges

PBS – Phosphate Buffered Saline

rGO – Reduced Graphene Oxide

ROS – Reactive Oxygen Species

SCGE – Single Cell Gel Electrophoresis

SOD – Superoxide Dismutase

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I. Introduction

Nanotechnology

Nanotechnology is the science that studies and develops technology at the atomic, molecular and macromolecular scale, leading to the controlled manipulation of structures and devices with domain dimensions below 100 nm (McNeil, 2005; Nowack & Bucheli, 2007). It exploits chemical, physical, electrical, and mechanical properties that emerge when matter is structured at the nanoscale (Buzea et al., 2007). Engineered nanoparticles have become an important class of new materials with several properties that make them very attractive for commercial development. Due to such a small scale of manipulation, the materials produced with the help of nanotechnology find their potential applications in many different fields, such as electronics, aerospace, computer industry, cosmetics and biomedical research (Fig. 1) (Medina et al., 2009; Hyuk et al., 2010).

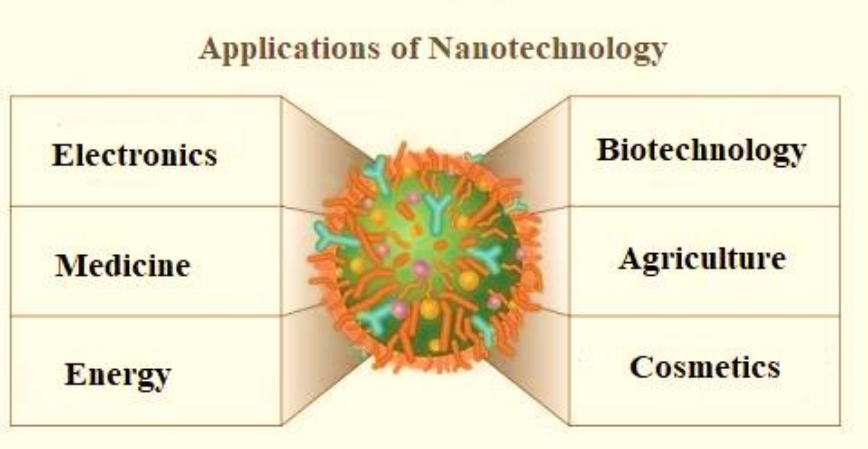


Figure 1. Applications of Nanotechnology.

An obvious advantage of nanotechnology is the ability to control the size of the resulting particles and devices. Nanoscale devices and components are of the same basic size as biological entities. Individual nanoscale particles can also provide remarkable analytical sensitivity in a variety of biological assays, in real time and without the use of radioisotopes (McNeil, 2005).

Although nanotechnology is a relatively young field, it is developing rapidly, thanks to a strong foundation of material science and engineering. Because of the potential of this technology there has been an increase in investment in nanotechnology research and development (Guzman et al., 2006). Despite of their unique advantages and applications in domestic and industrial sectors, use of materials with dimensions in nanometres has raised the issue of safety for workers, consumers and the environment (Sajid et al., 2015).

Nanomaterials Definition and Classification

In 2011, the European Commission defined a nanomaterial as "a natural, incidental or manufactured material containing particles in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm" (Fig. 2). Also, the "fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials" (European Commission, 2011).

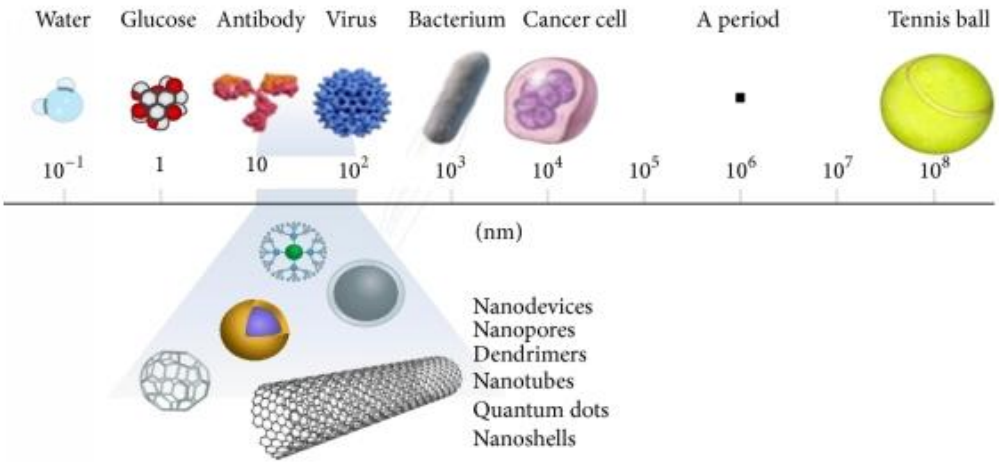


Figure 2. A size comparison of nanoparticle with other larger-sized materials. Adapted from Amin et al. (2014).

Nanoparticles (NPs) have unique physical and chemical properties resulting from the high amount of atoms present on the surface, their increased surface area to volume ratio and extremely small size (Ghosh & Paria, 2012). NPs are therefore considered substances that are less than 100 nm in size in more than one dimension. As can be seen in Figure 3, a nanoparticle is 100–10,000 times smaller than the size of a mammalian cell.

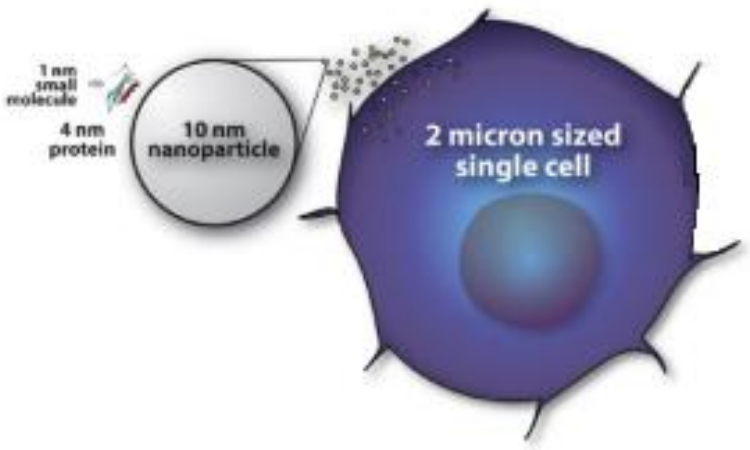


Figure 3. A mammalian cell is thousand times larger in volume and size compared to a 10 nm nanoparticle. Adapted from Hyuk et al. (2009).

NPs can be spherical, tubular, or irregularly shaped and can exist in fused, aggregated or agglomerated forms. They can be classified into natural and anthropogenic (engineered) particles (Nowack & Bucheli, 2007). Nanoparticles may be composed of minerals, metals or organic compounds and may also be mixtures of variable complexity depending on the method used to generate them (Andujar et al., 2011).

The interface between a nanoparticle and biological systems comprises three interacting components: the nanoparticle surface, the solid–liquid interface; and the solid–liquid interface's contact zone with biological substrates (Nel et al., 2009) (Fig. 4). These interactions are shaped by a great number of forces that could determine whether the nanoparticle is bioavailable and may participate in biocompatible or bio

adverse interactions. Processes as the formation of the protein corona, cellular contact, particle wrapping at cell surfaces, endocytosis and intracellular biocatalysis are determined by specific interactions (Nel et al., 2009).

The acquired properties of nanoparticles contribute actively for the interaction of nanoparticles with the biological medium. However, forces like long-range forces, which arises from van der Waals (attractive in nature) and electrostatic double layer interactions, and short range forces, which arise from charge, solvent interaction, steric hindrance and depletion are found at the nanoparticle media interface responsible for interaction with biomolecules (Nel at al., 2009).

The biophysicochemical influences on the interface between nanomaterials and biological systems are represented in table 1.

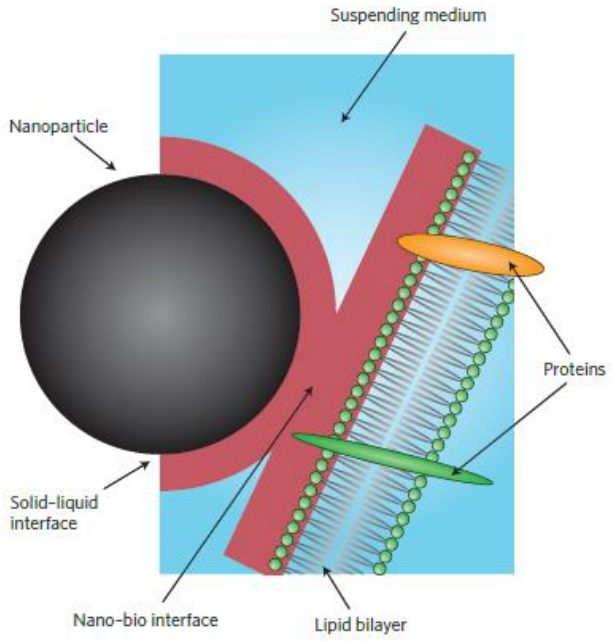


Figure 4. Representation of the interface between a nanoparticle and a lipid bilayer. Adapted from Nel et al. (2009).

Table 1. Main biophysicochemical influences on the interface between nanomaterials and biological systems (Nel et al., 2009).

Nanoparticle
Size, shape and surface area
Surface charge, energy, roughness and porosity
Valence and conductance states
Functional groups
Ligands
Crystallinity and defects
Hydrophobicity and hydrophilicity
Suspending media
Water molecules
Acids and bases
Salts and multivalent ion
Natural organic matter (proteins, lipids)
Surfactants
Polymers
Polyelectrolytes
Solid–liquid interface
Surface hydration and dehydration
Surface reconstruction and release of free surface energy
Ion adsorption and charge neutralization
Electrical double-layer formation, zeta potential, isoelectric point
Sorption of steric molecules and toxins
Electrostatic, steric and electrosteric interactions
Aggregation, dispersion and dissolution
Hydrophilic and hydrophobic interactions
Nano–bio interface
Membrane interactions: specific and nonspecific forces
Receptor–ligand binding interactions
Membrane wrapping: resistive and promotive forces
Biomolecule interactions (lipids, proteins, DNA) leading to structural and functional effects
Free energy transfer to biomolecules
Conformational change in biomolecules
Oxidant injury to biomolecules
Mitochondrial and lysosomal damage, decrease in ATP

Nanotoxicology

Nanotoxicology is a branch of toxicology that can be defined as the study of the toxicity of nanomaterials. Humans have been exposed to naturally formed nanoparticles throughout their evolution; however, this exposure has been increased due to arise of engineered manufactured nanomaterials (ENMs) (Medina et al., 2009).

Humans can be exposed to nanoparticles by several exposure routes, such as inhalation, ingestion, dermal penetration, injection or implantation for biomedical applications (Buzea et al., 2007).

In a context of work place exposure, inhalation is a major route of exposure and this can occur in different stages of the manufacturing cycle such as in the production of the nanomaterial or during its incorporation into final products. Exposure as a result of product disposal is also possible through the release of nanomaterials into watercourses (Maynard, 2012). Nanoparticles can be easily deposited in the lungs or on the skin, and have the ability to redistribute from their site of deposition. Agglomerates of nanoparticles larger than 100nm in diameter can also be inhaled, ingested or deposited on the skin, and may have the potential to express toxicity associated with their nanostructure (Oberdörster et al., 2005).

The routes of exposure to nanoparticles and its effects are illustrated in figure 5. Some of the diseases associated with inhaled NPs are asthma, bronchitis, lung cancer, and neurodegenerative diseases, such as Parkinson's disease. Presence of NPs in the gastrointestinal tract have been linked to Crohn's disease and colon cancer. Occurrence of arteriosclerosis, blood clots, arrhythmia and heart diseases are related to nanoparticles that enter the circulatory system. Autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis may occur due to exposure to some NPs (Buzea et al., 2007).

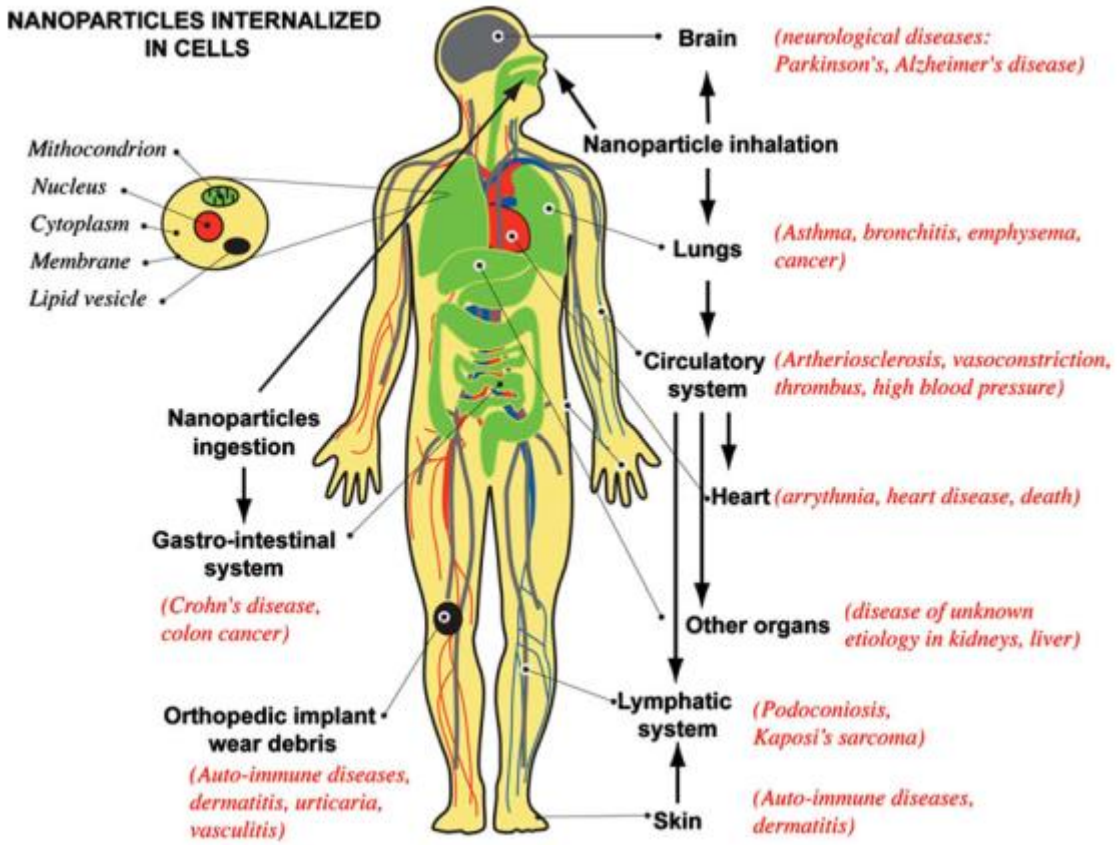


Figure 5. Schematics of human body with pathways of exposure to nanoparticles, affected organs and associated diseases. Adapted from Buzea et al. (2007).

Features such as NP size, shape, surface properties, composition, solubility, aggregation/agglomeration, particle uptake, and the presence of mutagens can influence the mechanisms of toxicity (Fig. 6). The smaller particles are, higher is the surface area they have per unit mass; and this property makes nanoparticles very reactive in the cellular environment. Therefore, any intrinsic toxicity of the particle surface will be enhanced (Donaldson et al., 2004). It is important to mention that the toxicity of any nanomaterial to an organism depends on the individual's genetic complement, which provides the biochemical tools needed to adapt and fight toxic substances (Buzea et al., 2007).

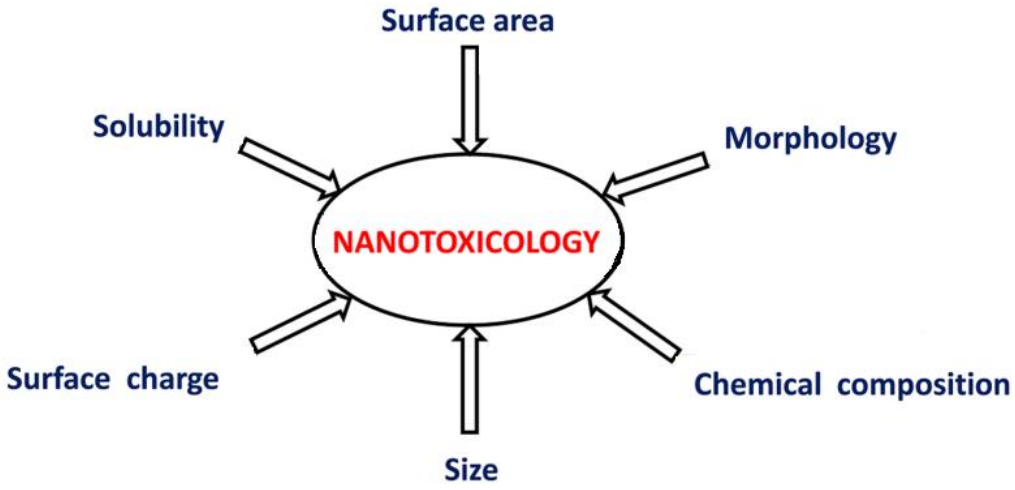


Figure 6. Physiochemical properties of nanomaterials leading to nanotoxicology.

Genotoxicity can be generated by direct interaction of nanoparticles with the genetic material, by indirect damage from NP-induced ROS, or by toxic ions released due to NPs solubilization. NPs that cross cellular membranes may be able to reach the nucleus through diffusion across the nuclear membrane or transportation through the nuclear pore complexes, and interact directly with DNA organised in chromatin or chromosomes (Magdolenova et al., 2014).

Some studies describe de ability of NPs to travel via the nasal nerves to the brain, as has been described for polio virus, and to gain access to the blood and other organs. They may not be detected by the normal phagocytic defences, allowing them to gain access to the blood or the nervous system. NPs can trigger ROS production in activated phagocytes (neutrophils, macrophages).

Graphene Nanomaterials

Graphene is a purely carbon-based, honeycomb-structured, one-atom thick layer of carbon atoms, bonded to another by sp^2 hybridization, resulting in a large surface area on both sides of the planar axis. Its most frequent form in nature is graphite, a mere stack of graphene layers held together by Van der Waals interactions (Fig. 7) (Geim and Novoselov, 2007; Novoselov, 2004).

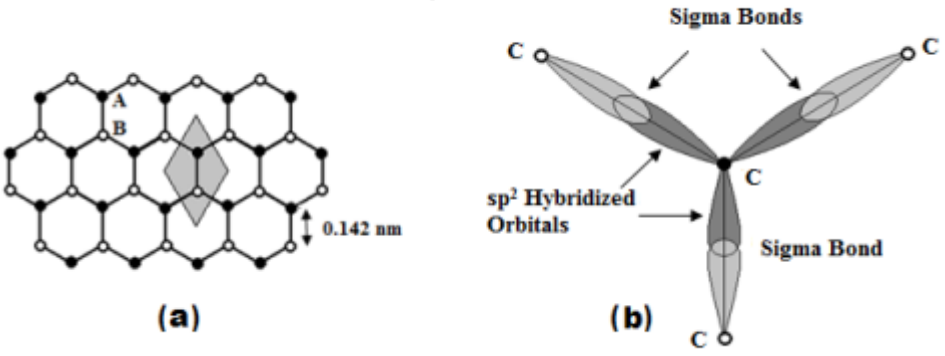


Figure 7. Structure of graphene. (a) Honeycomb structure of carbon atoms; (b) sp2 hybridized orbitals of carbon atoms symmetrically distributed. Adapted from Dash et al. (2014).

The theoretical existence of graphene was discussed over 60 years ago by Slonczewski and Weiss (1958). However, in 2004, Geim and Novoselov isolated single sheets of graphene by micromechanical cleavage of graphite and characterized their quantum electrodynamics (Novoselov et al., 2004).

Graphene and its derivatives, referred to as graphene family nanomaterials (GFNs), vary in layer number, lateral dimension, surface chemistry, defect density or quality of the individual graphene sheets, and composition or purity (Fig. 8) (Sanchez et al., 2012). Sanchez et al. (2012) proposed a systematic nomenclature for the derivatives of graphene and those materials are:

a) **Monolayer graphene** can be isolated from graphite by repeated "mechanical exfoliation" and is the material that has attracted the most interest due to its unique electronic properties;
b) **Few-layer graphene (FLG)** is defined as flake-like stacks of 2–10 graphene layers. It was originally a by-product of, or precursor in, the fabrication of monolayer graphene;
c) **Ultrathin Graphite** is defined as material with thickness greater than 10 sheets (3–5 nm) but less than 100 nm;
d) **Graphene oxide (GO)** is a highly oxidized form of chemically modified graphene, produced by harsh oxidation of crystalline graphite. The structure of GO consists of single-atom-thick carbon sheets with carboxylate groups on the periphery;
e) **Reduced graphene oxide (rGO)** is the product of treating GO under reducing conditions, which include high-temperature thermal treatment and chemical treatments with hydrazine (N_2H_4) or other reducing agents. The goal of GO reduction is often done to restore electrical conductivity;
f) **Nano-GO** is a term sometimes used to describe graphene oxide of small lateral dimension, typically less than 100 nm and often below 20 nm. These materials have typically been used in biological applications, because small size facilitates cell entry and dispersion stability.

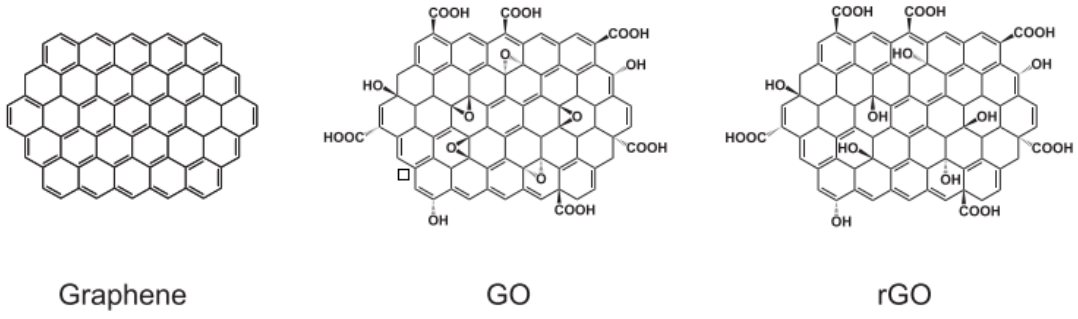


Figure 8. Structure of Graphene, Graphene Oxide (GO) and Reduced Graphene Oxide (rGO). Adapted from Zhang et al. (2016).

The properties of GFNs most important for their effects in biological systems include surface area, layer number, lateral dimension, surface chemistry, and purity.

Surface Area

Surfaces play a central role in the biological interactions of nanomaterials (Nel et al., 2009). Small nanoparticles (<10 nm) have a significant fraction of their atoms exposed on their surfaces. In case of monolayer graphene, every atom lies on the surface, and in fact each atom is exposed to the surrounding medium on two sides. The surface areas of other GFNs decrease as layer number increases. Due to the high surface area of GFNs, physical adsorption or catalytic chemical reaction will be important in the biological response to these materials (Sanchez et al., 2012).

Layer Number

The number of graphene layers in a GFN determines specific surface area and bending stiffness (Sanchez et al., 2012). The adsorptive capacity for biological molecules increase significantly as layer number decreases. Stiffness is reported to be important in the pathological response to fibers and carbon nanotubes (Poland et al., 2008).

The thinnest materials, such as monolayer graphene or GO, are quite deformable by weak forces such as water surface tension. The multilayer materials in contrast may act as rigid bodies during their cellular interactions.

Lateral dimension

Lateral dimension determines the maximum dimension of the material, which is relevant for cell uptake, renal clearance, blood-brain barrier transport and other biological interactions that depend on particle size. Lateral dimension affects the

population of receptors needed for uptake, and also the size of the endosome or lysosome into which the material must be packaged within the cell. The lateral sizes of GFNs span orders of magnitude, from nano-GO at 10 nm (the size of some proteins) to >20μm (larger than most cells) (Sanchez et al., 2012).

Surface chemistry

The graphene family includes materials with widely varying surface chemistry. Graphene oxide surfaces are in part hydrophobic with hydrophilic regions capable of hydrogen bonding and metal ion complexing, and contain negative charges on edge-sites associated with carboxylate groups. The pristine graphene surface, in contrast, is hydrophobic and capable of biochemical reactions primarily at edge or defect sites. Reduced graphene oxide is intermediate in hydrophilicity and in basal reactivity, since it contains basal vacancy defects produced during oxygen removal (Sanchez et al., 2012).

Purity

Graphene nanomaterials typically do not contain residual metal catalysts. However, some of them may contain residual intercalants, chemical additives used to separate the layers in the bulk graphite feedstock and have not been fully removed by washing. The reagents used in various GFN syntheses include permanganate, nitrate, sulphate, chromate, peroxide, persulfate, hydrazine and borohydride and associated cations, typically potassium, sodium, or ammonium (Sanchez et al., 2012).

Graphene applications

Graphene has been used in diverse nanobiotechnological applications, such as in the environment, biomedicine and biotechnology.

Graphene nanomaterials possess potential in the biomedical field as biosensors, tissue scaffolds, carriers for drug delivery or gene therapy, antibacterial agents, stem cell technology, photo thermal therapy and bio-imaging (Gulzar et al., 2017; Ou et al., 2017; Sanchez et al., 2012). In the last years studies have been carried on the delivery of anticancer drugs, genes and peptides via graphene derivatives. Recent studies suggest that graphene and GO are able to hasten the growth, differentiation, and proliferation of stem cells, and hence possess prodigious potential in tissue engineering, regenerative medicine, and other biomedical fields (see Gulzar et al., 2017).

Numerous types of graphene also present other properties such as high Raman scattering intensity, large absorption cross section in the NIR (Near infrared radiation) region, sharp photoacoustic contrast with the NIR incident beam, which all are indispensable properties for bio-imaging (Gulzar et al., 2017).

Although the application of graphene may provide consistent improvements or possible revolutions in the biomedical area, its use is not without risk to human health; therefore, a deeper level of nanotoxicological and human safety studies are required (Seabra et al., 2014).

Toxicity of Graphene Nanomaterials

GFNs present in biomedical and non-biomedical products manifested potential toxicity to humans, animals, and cells (Ou et al., 2017). Due to their size graphene nanoparticles can cross the physiological barriers by different exposure routes, for example, inhalation, ingestion, dermal penetration, or implantation for

biomedical applications. The major exposure route for GFNs in the working environment is airway exposure.

The properties of graphene nanomaterials, such as shape, size, concentration, lateral dimension, surface structure, functional groups, purity and protein corona influence toxicity in biological systems (Lalwani et al., 2016; Sanchez et al., 2012; Seabra et al., 2014; Yang et al., 2013).

GFNs get into the cell through different ways which lead to several reactions. The main mechanisms proposed by Ou et al. (2016) for the cytotoxicity of GFNs are: physical destruction, oxidative stress, mitochondrial damage, DNA damage, inflammatory response, apoptosis, autophagy and necrosis. A schematic of the main mechanisms of GFNs cytotoxicity is illustrated in Figure 9.

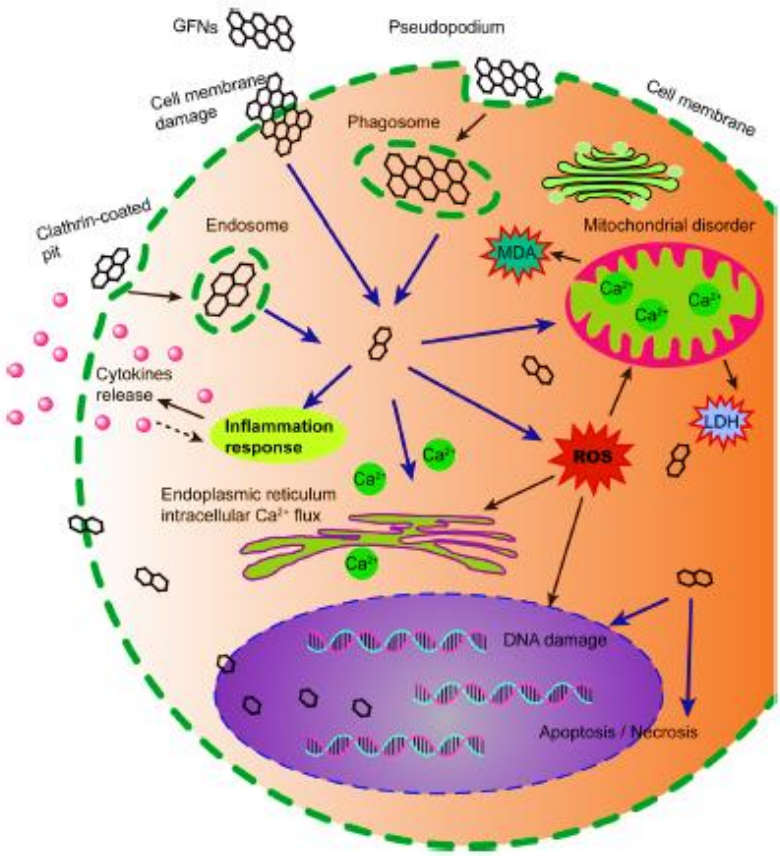


Figure 9. The possible mechanisms of GFNs cytotoxicity. Adapted from Ou et al. (2016).

Internalization

Shape, size and curvature of nanomaterials greatly influence the internalization of nanomaterials inside a cell.

Graphene and graphene-related nanomaterials are primarily internalized into cells via endocytosis. The internalization of graphene into cells is known to be related to the cell type. Macropinocytosis seems to be a general internalization process in three different cell lines (Saos-2 osteoblasts, HepG2 hepatoma cells, and RAW 264.7 macrophages) (Fig. 10). The internalization of graphene nanomaterials into cells is strongly influenced by the particle size and surface chemistry. Low concentrations of graphene nanomaterials show little or no toxicity in mammalian cells, however high concentrations of graphene alter the dynamics and integrity of the plasma membrane during their internalization, and induce cell death (Zhang et al., 2016).

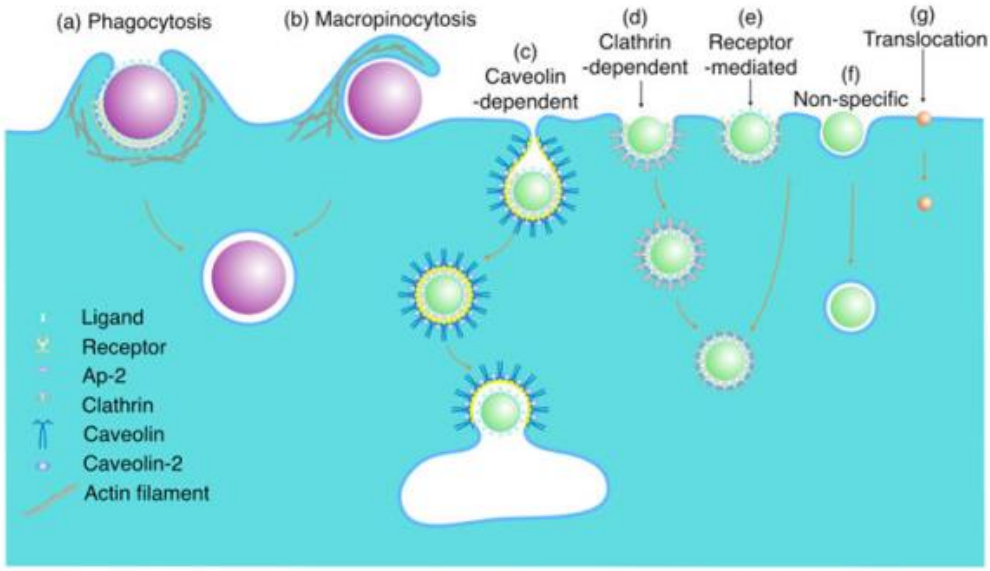


Figure 10. The possible internalization pathways of nanoparticles. Adapted from Zhang et al. (2015).

ROS Production

Oxidative stress arises when there is a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant enzymes, including catalase, superoxide dismutase (SOD), or glutathione peroxidase (GSH-PX) (Ou et al., 2016). Pristine graphene increase intracellular ROS in RAW 264.7 cells which is the first step in the mechanisms of carcinogenesis, ageing, and mutagenesis (Li et al., 2012). Oxidative stress had a significant role in GO-induced acute lung injury, and the inflammatory responses caused by oxidative stress often emerged upon exposure to GFNs (Ou et al., 2016). The activity of SOD and GSH-PX decreased after exposed to GO in a time- and dosage-dependent manner (Zhang et al., 2010).

Mitochondrial damage

Mitochondria are energy production centres, and the mitochondrial membrane potential (MMP) is crucial to ATP synthesis. Pristine graphene induced cytotoxicity in RAW 264.7 cells through the depletion of the MMP (Li et al., 2012).
GO caused the disturbance of the mitochondrial structure and function, as characterized by a decrease in the mitochondrial membrane potential and the dysregulation of mitochondrial Ca^{2+} homeostasis (Lammel et al., 2013). Direct influence of GFNs in cell mitochondrial activity can cause apoptosis and/or cell necrosis (Park et al., 2015; Shekaramiz, 2012).

DNA damage

Due to its small size, high surface area and surface charge, GFNs may possess significant genotoxic properties and cause severe DNA damage.
Carbon nanotubes (CNT) - allotropes of carbon with a cylindrical nanostructure - are capable of increasing chromosome and DNA damage as assessed by the

cytokinesis-blocked micronucleus and comet assays, respectively, in RAW 264.7 murine macrophages (Migliore et al., 2010).

GO can elevate the expression of p53, Rad51, and MOGG1-1, which reflect chromosomal damage, and decrease the expression of CDK2 and CDK4 by arresting the cell cycle transition from the G1 to the S phase in various cell lines. (Liu et al., 2013). GO may induce chromosomal fragmentation, DNA adducts and point mutations by promoting oxidative stress or triggering inflammation through the activation of intracellular signalling pathways such as MAPK, TGF-β and NF-κB (Chatterjee et al., 2014; Liu et al., 2013; Jarosz et al., 2015). GO caused DNA damages in HEK293T cells and induced base excision repair pathway in both HEK293T cells and zebrafish embryos (Lu et al., 2017).

Graphene-family nanomaterials (GFNs) possibly caused DNA damage to human bronchial epithelial cells by affecting nucleotide excision repair and non-homologous end joining repair systems (Chatterjee et al., 2016).

Apoptosis

Apoptosis is a genetically regulated process leading to the death of cells. It is a coordinated process that can be triggered through two different pathways, the death-receptor pathway (triggered by members of the death-receptor superfamily) and the mitochondrial pathway (in response to internal DNA damage) (Li et al., 2012). Wang et al. (2011) demonstrated that graphene oxide could produce cytotoxicity and can enter human lung fibroblasts cytoplasm and nucleus, inducing apoptosis at doses above 20 μg/ml after 24 h. rGO induced cell death in glioma cells mostly through the apoptosis pathway (Jaworski et al., 2015). Exposure of Raw 264.7 cells to pristine graphene caused an increase of apoptosis by activation of the mitochondrial pathway (Li et al., 2012). GFNs also had pro-apoptotic effects in cells (Ou et al., 2016).

Graphene can increase EGFR (epidermal growth factor receptor) expression in lung epithelial cells, which mediate several cell pathways that include enhancing the apoptosis progress (Fig. 11) (Tsai et al., 2017).

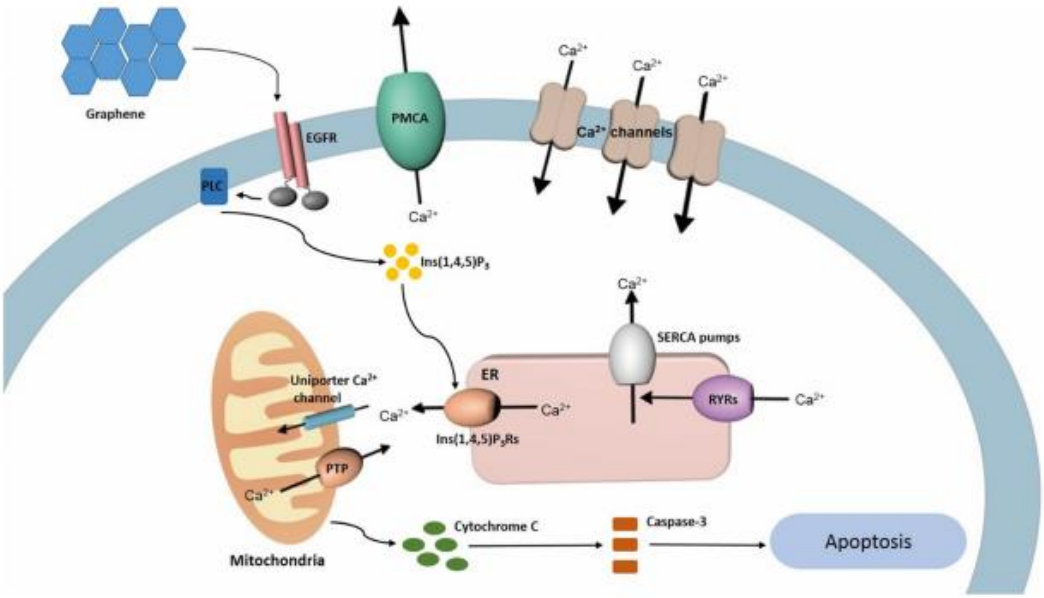


Figure 11. The model of how graphene induces EGFR activation. Adapted from Tsai et al. (2017).

Necrosis

Necrosis can be defined as cell death caused by loss of membrane integrity, intracellular organelle swelling and adenosine triphosphate (ATP) depletion leading to an influx of calcium (Cullen, 2010).

The exposure of cells to pristine graphene caused necrosis at high doses (50 mg/mL) (Li et al., 2012). GO treatment was revealed to induce necrosis in macrophages by activating TLR4 signalling and subsequently partly triggering autocrine TNF-α production (Qu et al., 2013).

In summary, many studies have discussed representative mechanisms of GFNs toxicity involving four signalling pathways: TLRs, TGF-β, TNF-α and MAPKs. These four signalling pathways are correlative and cross-modulatory, making the mitochondrial damage, apoptosis and other mechanisms independent and yet connected to each other. Additionally, oxidative stress appears to play the most important role in activating these signalling pathways (Ou et al., 2016).

II. Assay Techniques

In vitro cell culture

The removal of cells from an animal or plant and their subsequent growth in a favourable environment is called cell culture. A primary cell culture may be obtained by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate (Fig. 12). Primary culture refers to the stage of the culture after the cells are isolated from the tissue or organ and attached, divided and proliferated under the appropriate conditions until they reach confluence. Then, they must be subcultured to give them room for continued growth by transferring them to a new vessel with fresh growth medium. This constitutes a "passage" and the daughter cultures so formed are the beginnings of a cell line (Freshney, 1993; Helgason & Miller, 2004).

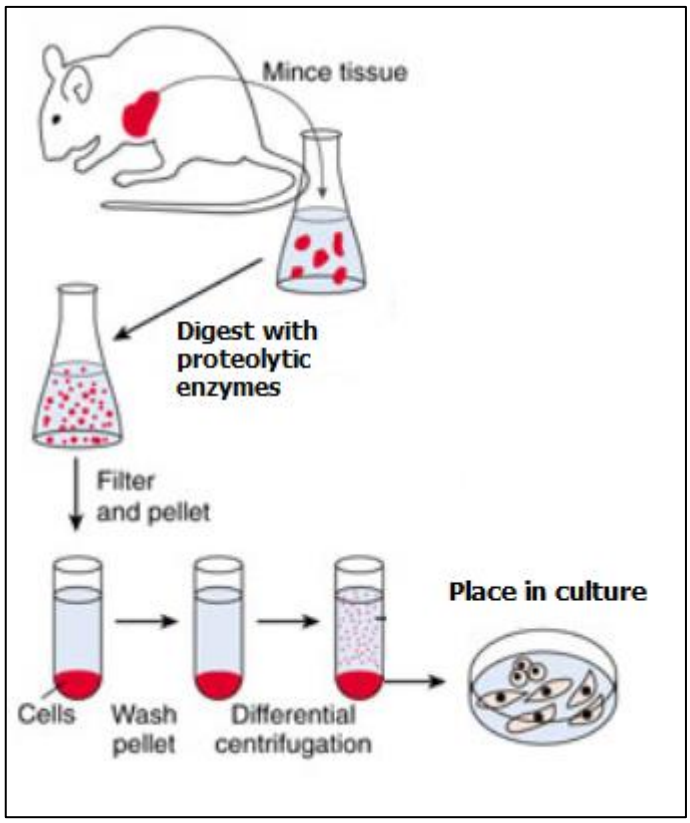


Figure 12. Primary cell culture. Adapted from Wagner & Hewlett (2004).

Two basic systems are used for growing cells in culture, as monolayers on an artificial substrate (i.e., adherent culture) or free-floating in the culture medium (suspension culture). Cells in culture can be divided into three categories based on their morphology (Fig. 13):

- Epithelial-like: cells that are attached to a substrate and appear flattened and polygonal in shape;

- Lymphoblast-like: cells that do not attach normally to a substrate but remain in suspension with a spherical shape;

- Fibroblast-like: cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures.

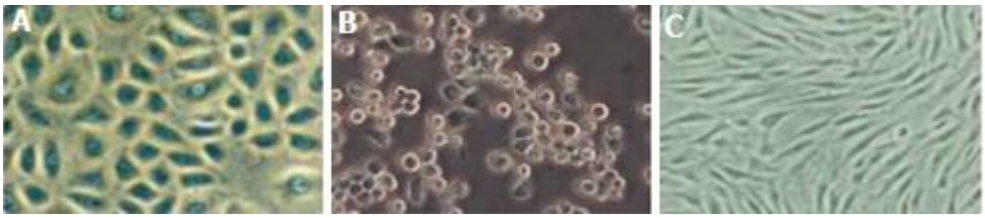


Figure 13. A - Epithelial-like cells; B - Lymphoblast-like cells; C - Fibroblast cells.

The characteristics of *in vitro* cultured cells are determined by both their origin (liver, heart, etc.) and how they adapt to the culture conditions. Biochemical markers can be used to determine if cells are still carrying the specialized functions they presented *in vivo*. Frequently, these characteristics are either lost or changed due to the artificial environment. Some cell lines will eventually stop dividing and show signs of aging. Other lines are, or become immortal; these can continue to divide indefinitely. Continuous culture of cell lines can lead to the accumulation of unwanted karyotype alterations or the outgrowth of clones within the population. In addition, continuous growth increases the possibility of cell line contamination by bacteria or other unwanted organisms.

In vitro cell culture is an invaluable tool for investigators in numerous fields. It facilitates analysis of biological properties and processes that are not readily accessible at the level of the intact organism. This is one of the main tools used in cellular and molecular biology, allowing the study of the normal physiology and biochemistry of cells, the effects of chemicals on the cells and its potential mutagenic and carcinogenic effects. It is also used in drug screening and development, and large scale manufacturing of biological compounds, such as vaccines, therapeutic and proteins. The major advantage of using cell culture is the consistency and reproducibility of results that can be obtained from using clonal cells.

The major requirement of a cell culture laboratory is the need to maintain an aseptic work area that is restricted to cell culture work. Aseptic technique, designed

to provide a barrier between the microorganisms in the environment and the sterile cell culture, depends upon a set of procedures to reduce the probability of contamination from these sources. The elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling (Freshney, 1993; Helgason & Miller, 2004).

Assays for genotoxicity evaluation

Cytokinesis-blocked micronucleus cytome assay

The DNA is an intrinsically reactive molecule and is highly susceptible to chemical modifications by endogenous and exogenous agents that can impact health and modulate disease-states, therefore the study of DNA damage is an essential part of genetic toxicology.

The cytokinesis-blocked micronucleus assay (CBMN) is one of the most commonly used methods for measuring DNA damage and cytotoxicity. DNA damage is scored specifically in once-divided binucleated (BN) cells and include micronuclei (MNi), nucleoplasmic bridges (NPBs), and nuclear buds (NBUDs) (Fig. 14). MNi are small extranuclear bodies resulting from chromosome fragments or whole chromosomes lagging behind during anaphase. NPBs originate from dicentric chromosomes that may be caused by misrepair of double strand DNA breaks or telomere end fusions (Fenech, 2007). Cells that have completed one nuclear division are blocked by cytochalasin-B (Cyt-B), an actin polymerization inhibitor, and are consequently identified by their binucleated appearance (Fenech, 2000).

This method is an efficient assay of DNA damage, chromosomal instability, mitotic abnormalities, cytostasis and cell death. CBMN assay allows the measure of many events of cellular and nuclear dysfunction such as unrepaired chromosome breaks, DNA misrepair, telomere end fusions, chromosomal instability, altered mitotic activity and/or cytostasis and cell death by necrosis or apoptosis.

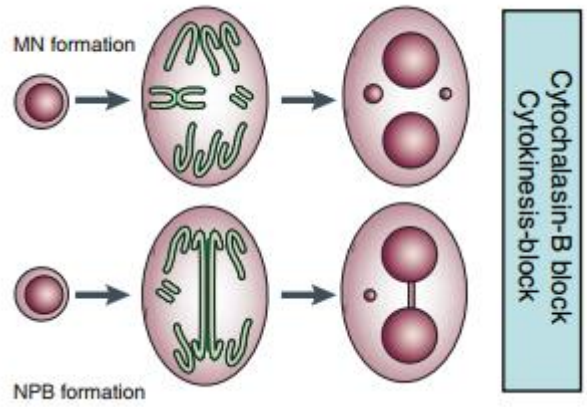


Figure 14. MN and NPB formation in cells during nuclear division. These events can only be observed in BN cells after cytokinesis blocking with Cyt-B. Adapted from Fenech (2007).

Figure 15 shows a representative example of the possible biomarkers present in cells scored in the CBMN assay. The CBMN assay is the preferred method for measuring MNi in cultured human and mammalian cells because scoring is specifically restricted to once-divided BN cells, which are the cells that can express MNi.

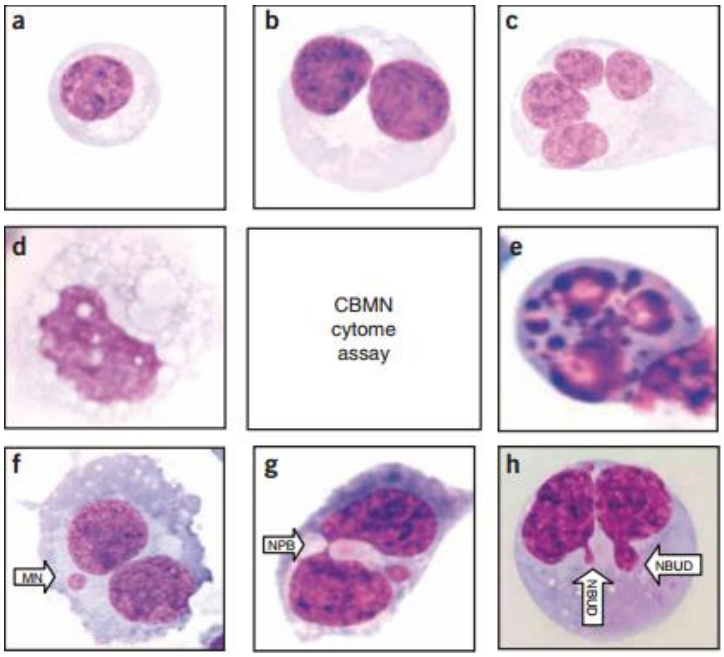


Figure 15. Photomicrographs of the cells scored in the CBMN assay. (a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) early necrotic cell; (e) late apoptotic cell; (f) BN cell containing one MNi; (g) BN containing an NPB (and a MN); (h) BN cell containing NBUDs. Adapted from Fenech (2007).

The Comet Assay

The comet assay or single cell gel electrophoresis (SCGE) assay is a gel electrophoresis–based method that can be used to measure DNA damage in individual eukaryotic cells (Olive & Banáth, 2006), with applications in genotoxicity testing, human biomonitoring and molecular epidemiology, as well as fundamental research in DNA damage and repair (Collins, 2004).

This assay is a rapid, sensitive and relatively simple method and combines biochemical techniques for detecting DNA single strand breaks with the single cell approach typical of cytogenetic assays.

The Comet Assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells (Fig. 16). It should be noted that DNA lesions consisting of strand breaks after treatment with alkali either alone or in combination with certain enzymes (e.g. endonucleases) increases DNA migration, whereas DNA-DNA and DNA-protein cross-links result in retarded DNA migration compared to those in concurrent controls (Tice et al., 2000). The comet assay is most commonly applied to animal cells, whether in culture or isolated from the organism (Collins, 2004).

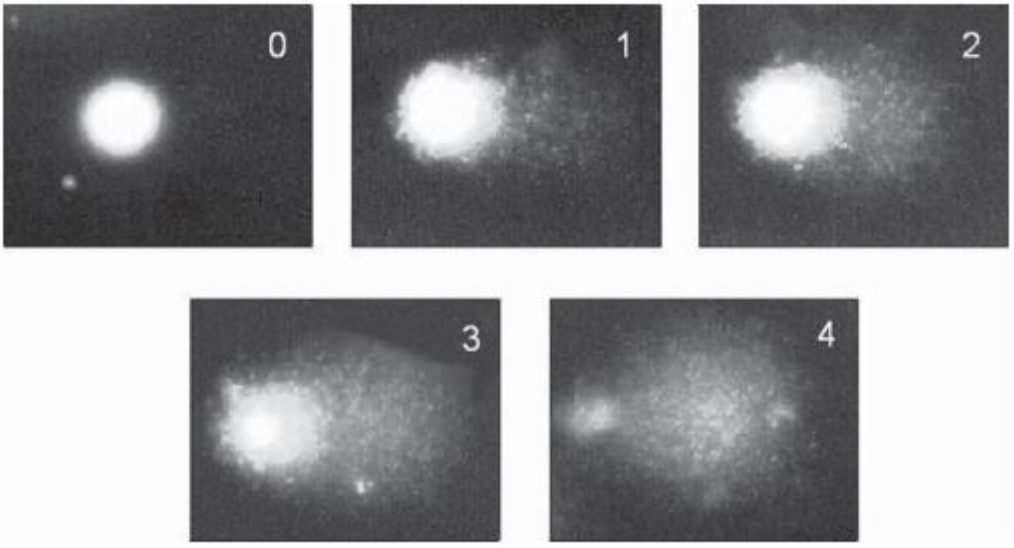


Figure 16. Images of comets (from lymphocytes), stained with DAPI. They represent 5 classes from 0 (no tail) to 4 (almost all DNA in tail) (Collins, 2004).

III. Raw 264.7 cell line

Potential target cells in the lungs following inhalation of GFNs include alveolar macrophages, lung epithelial cells, and fibroblasts in the interstitium of the alveolar walls (Sanchez et al., 2012). Macrophages play a key role in the cellular response and represent the active line of defence against inhaled particles that deposit in the lungs. Studies have been conducted to evaluate their property of initiating and propagating inflammatory reactions (Kagan et al., 2006; Oberdörster et al., 2005).

The macrophage cell line RAW 264.7used in the present study was isolated from *Mus musculus*, which are derived from Abelson murine leukaemia virus-induced tumour. This lineage is considered a representative model for the study of lung responses to inhaled NPs and ultrafine particles (Fig. 17). This cell line is easy to propagate, highly efficient for DNA transfection, sensitivity to RNA interference, and supports replication of murine noroviruses (ATCC, 2016).

The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) and to make the complete growth medium, it is necessary to supplement it with fetal bovine serum (FBS). The requirements for cell growth are atmosphere of 95% oxygen and 5% carbon dioxide at a temperature of 37ºC to ensure the conditions necessary for growth.

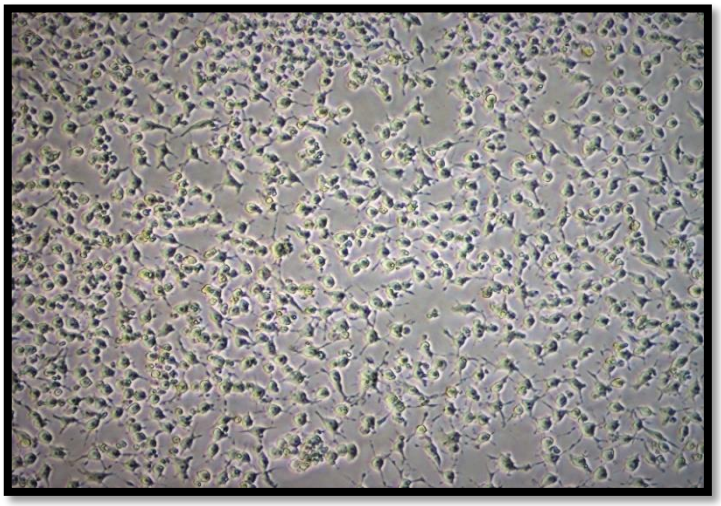


Figure 17. RAW 264.7 cells in culture.

IV. Aims

The general aim was to perform a comprehensive study on the genotoxicity of seven graphene nanomaterials to macrophage RAW 264.7 cells using carbon black as reference, to establish a toxicity ranking.

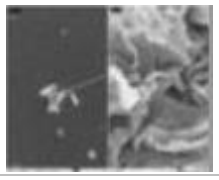
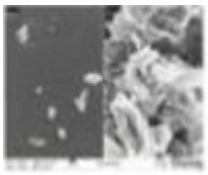
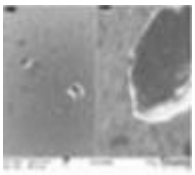
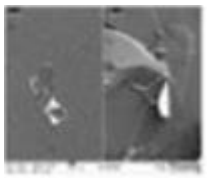
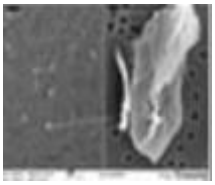
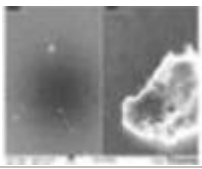
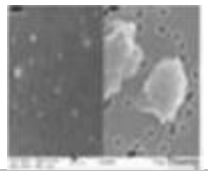
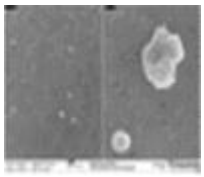
V. Materials and Methods

Test Materials and Characterization

For the current study, 7 GFNs compounds were selected (P1-P7) and carbon black (P8) was used as reference (Table 2):

P1. Single-Layer Graphene Powder;
P2. Single-Layer Graphene from factory series;
P3. Carboxyl Graphene;
P4. Graphene Nanoplatelets;
P5. Graphene Oxide Powder (S Method);
P6. Graphite Oxide Powder;
P7. Reference Pristine Graphene Nanoplatelets;
P8. Reference Carbon Black particles.

Table 2. Detailed information of the several nanomaterials used in this study.

Sample	Source Product	Diameter (µm)	Thickness	Specific surface (m^2/g)	SEM image	Preparations/ Properties
P1	ACS GN1P 0005	~5	2-10	278 (400-1000)		Thermal exfoliation reduction + Hydrogen reduction
P2	ACS GN1PF	0.5-5	2-10	620 (650-750)		1-5 atomic layer graphene nanosheets
P3	ACS GN1PF 010	1-5	0.8-1.2	1.5		1 – Modified Hummer's method to make graphene oxide; 2 – Convert –OH and C-O-C into –COOH. Carboxyl ratio: 5%
P4	ACS GNNP 0051	~5	2-10	15 (20-40)		Stacks of multi-layer graphene, with a high aspect ratio, width to thickness
P5	ACS GNOS 0010	1-15	0.8-1.2	5.2 (5-10)		Stauden-maier method; oxygen content: 35%
P6	ACS GTOP 0002	0.5-5	1-3	2.7		Modified Hummer's method: oxygen content: 35%
P7	AVANZARE	2	3	195 (70)		No XPS (low defects by RAMAN) all CS1 carbons. 8±0.5 atomic layer graphene
P8	Evonik Degussa	14	-	317 (337)		Specified as >99% pure carbon black

Cell Line Culture

For this study, the RAW 264.7 cell line was used as a model. Cells were cultured in 75 cm^2 culture flasks with 10mL Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% fungizone. The cell cultures were maintained in a humidified atmosphere (5% CO_2) at 37°C. The cells were observed every day under the inverted microscope (Nikon®Eclipse TS100) to check if there were contaminations and to evaluate their morphology and confluence (% of cell coverage on the surface area of the flask). The cells were subcultured every 2-3 days, when cultures reached about 70-80% confluence. The medium was removed and the cells were washed with phosphate buffered saline-PBS. To harvest cells from culture flasks a cell scraper was used, and the cell suspension was resuspended in culture medium and counted using a Neubauer chamber. The last step of the process was the seeding of cells in new flask with complete culture medium at the conditions already described. Aseptic techniques suitable for cell culture were followed throughout the experiments.

Exposure to GFNs

Cells were seeded in 12 and 6 well plates and incubated for 24h at 37°C, 5% CO_2, to adhere. After that period, the culture medium was removed and replaced for the same amount of the appropriate dilutions of GFNs. The concentrations for exposure were defined based in previous studies in which RAW 264.7 cells were exposed to a range of 0.00-50.00 $\mu g/cm^2$ GFNs for 24h and 48h and cell viability was assessed by AlamarBlue (AB) and lactate dehydrogenase (LDH) assays (Menezes et al, 2017).

The benchmark dose 30 (BMD30) and 0.5 BMD30 of each GFN for 24h were selected for the cell exposure in the subsequent assays and are shown in detail in table 3 (Menezes et al., 2017).

Table 3. Concentrations of particles per area.

Sample	Concentration for BMD30 (µg/cm^2)	Concentration for 0.5BMD30 (µg/cm^2)
P1	46.8	23.4
P2	50	25
P3	22	11
P4	25.3	12.7
P5	29.4	14.7
P6	23.6	11.8
P7	50	25
P8	50	25

Cytokinesis-blocked micronucleus cytome assay

To assess the genotoxic effects of GFNs in RAW 264.7 macrophages, the CBMN assay was performed as follows. Before seeding the cells, a coverslip was placed in each well of a twelve-well plate. The appropriate dilutions were made and a suitable quantity of cells (2 x 10^4 cells/cm^2) was seeded into each well, and two paired, independent cultures for each concentration were prepared. After 24 hours, the macrophages were treated with each GFN as described earlier. Cells exposed to methanesulfonate (MMS) at 25µg/mL were used as a positive control.

Forty-four hours after the initial preparation of the cultures, cytochalasin B was added to each test well to a final concentration of 4 µg/ ml to block cytokinesis (Fenech, 2007) followed by an incubation period of 28 hours. Then the medium was removed and each well was washed with 1mL of PBS. Absolute methanol cooled at 4°C (2mL) was added to each well to fix the cells (10min). The slides were stained with 1% acridine orange for 30 seconds, and mounted with one drop of cold distilled water. The slides were observed under the fluorescence microscope with an excitation filter of 450-490 nm and a barrier filter of 520 nm.

VI. Results and discussion

Graphene family nanomaterials have drawn much scientific attention and technological interest since their discovery due to their unique electronic and mechanical properties (Jastrzębska et al., 2012).

Previous studies with different cellular or animal model have demonstrated that some of the graphene derivatives induced significant cytotoxicity and genotoxicity. The interactions of graphene with cells, proteins, and other biomolecules are influenced by its physiochemical properties such as shape, size, surface charge, stability and purity and contribute to the differential toxicity observed (Park et al., 2015; Wang et al., 2013). Also for graphene-based materials it was reported that the different physicochemical properties result in different cellular toxicities (Ou et al., 2017; Zhang et al., 2014). *In vitro* toxicity investigation suggests that graphene exhibit dose-dependent toxicity to mammalian cells (Jastrzębska et al., 2012).

Figure 18 shows the effect of exposure for 24h of graphene nanoplatelets at 0.5 BMD30dose to RAW264.7 cells. Microscopic observations of cell cultures showed decreased cell confluence and increased the number of dead cells.

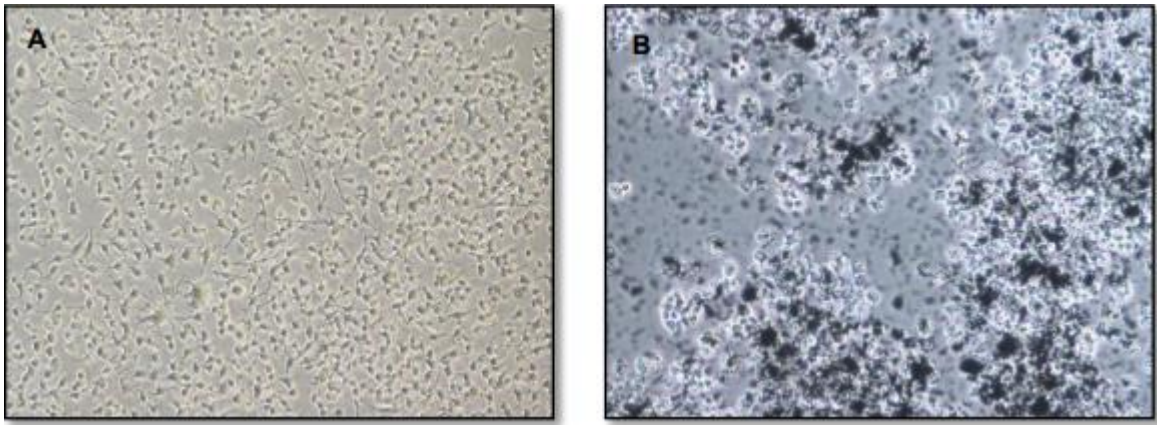


Figure 18. Microscopic images of RAW264.7. A – RAW264.7 cells in control conditions, B - Microscopic images of RAW264.7. B -RAW264.7 cells to 0.5BMD30 of P4 after 24 hours of exposition.

The main mechanism of graphene toxicity is associated with the generation of intracellular reactive oxygen species that cause damage to proteins and DNA leading to cell death via apoptotic or necrotic pathways (Li et al., 2012; Ma et al., 2014; Zhang et al., 2012). Some studies have shown that GFNs could cause epigenetic and genomic changes that might stimulate physical toxicity and carcinogenicity (Soldano et al., 2010; Max Costa & Yao, 2013).

In the present thesis, preliminary studies on the effects of several GFNs on the micronuclei (MNi) induction (assessed by the cytokinesis-block micronucleus cytome assays, CBMN) were performed. Cells exposed to methanesulfonate (MMS) at 25 µg/mL were used as a positive control. In the first analysis of the slides it was verified that there were many multinucleated cells instead of the expected binucleated cells, which made it difficult to visualise the cells. This may have happened due to the concentration of cytochalasin B added to each test well. The time of exposure may not have been adequate either. An adjustment to time and concentration of cytochalasin B should be made in future studies with the Raw 264.7 cell line. Evaluation and interpretation of results were not performed.

VII. Conclusions - Future Perspectives

Presently, the literature is insufficient to draw conclusions about the potential hazards of GFNs. Considering that humans are usually exposed to GFNs, especially at their manufacture, it is important to determine if or which GFNs are more cytotoxic.

For all future studies of GFNs toxicity it is very important a detailed physiochemical characterization since these physicochemical factors influence the toxicity and biocompatibility of GFNs. The selection of cell lines is of vital importance because some of them tend to be sensitive or resistant depending upon their genetic background. The same graphene nanoparticles can cause different reactions depending on their various cells origins. More specific signalling pathways in the mechanism of GFNs toxicity need to be discovered and elucidated. A deeper study on the mechanisms underlying cytotoxicity by GFNs could be performed, for instance, the analysis of cytochrome c to better understand the relation between mitochondrial physiology and the release of cytochrome in apoptosis initiation. ATP measurements, protein expression and autophagy studies are also recommended.

For all types of graphene nanoparticles, it is important to investigate and critically evaluate the potential short- and long-term health risks and toxicity hazards after acute, sub-acute and chronic exposures using *in vitro* and *in vivo* models.

Finally, there is a need for systemic solutions, monitoring and recording of potential hazard of GFNs as well as finding timely responses in order to achieve safety for human health and environment.

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